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[54]发明名称 甲型肝炎疫苗的生产方法

[57]摘要

本发明提供了制备甲型肝炎 L-A-1 减毒病毒株 (CCTCC NO, V92004) 和以所说的病毒株为毒种大规模工业化生产甲型肝炎疫苗的方法。

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权利要求书

1.一种制备甲型肝炎减毒活病毒株的方法，该方法包括提供含 L-A-1 病毒株的甲型肝炎急性期病人粪便样品的悬浮液，以此病毒悬浮液感染人胚肺二倍体细胞，当达到病毒增殖高峰后制备细胞提取物，并以该提取物为种子病毒来源用同一细胞基质进行连续传 10-25 代减毒，得到甲型肝炎 L-A-1 减毒活病毒株（CCTCC No.V92004）。

2.根据权利要求 1 的方法，其中所说的人胚肺二倍体细胞和所说的同一细胞基质是指人胚肺二倍体细胞单层。

3.根据权利要求 1 的方法，其中所说的连续传代减毒是用猴猿为检测对象监测病毒减毒水平。

4.一种以甲型肝炎 L-A-1 减毒活病毒株为毒种大规模工业化生产甲型肝炎疫苗的方法，该方法包括用旋转培养法培养人胚肺二倍体细胞，用伊尔氏液洗细胞表面，然后重接种 L-A-1 减毒活病毒并置 35℃下培养，约 4 周后，换成 199 综合培养液继续培养。

5.根据权利要求 4 的方法，其中所说的继续培养的时间为 2-5 天。

本发明涉及甲型肝炎疫苗，特别是涉及制备甲型肝炎 L-A-1 减毒病毒株和以该病毒株为毒种生产甲型肝炎疫苗的方法，以及该疫苗在预防甲型肝炎病毒感染中的应用。

甲型肝炎是一种由自然界广泛存在的甲型肝炎病毒（HAV）引起的全球性传染病，全世界约有 40 亿人口受到该疾病的威胁。在包括中国在内的发展中国家，由于人口众多、社会经济落后及卫生条件低下等原因，时有甲型肝炎的大规模暴发或局部流行。在经济发达的美国，每年也有高达 10,000 例肝炎病人与此类病毒感染有关，甲型肝炎的发病率占临床肝炎总病例数的 15～20%，据粗估计，在中国有近 5 亿人口受到甲型肝炎的威胁，每 10 万人口中约有 200～300 人遭受感染。上海市 1983 年和 1988 年两次甲型肝炎大流行，给当地人民的健康和国民经济发展带来严重损害。对此，至今人们仍然心有余悸。面对这样的现实，迫切要求发展有高特异性和安全的、可适于临床应用的甲型

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肝炎疫苗，对易感人群进行大规模免疫接种，以大幅度降低甲型肝炎发病率并有效地控制其暴发性流行。

七十年代中期以来，许多研究者致力于甲型肝炎减毒活疫苗或灭活疫苗的研究。如美国专利 4,164,566 号公开了在亚人类灵长目动物（如狨猴）细胞培养物中连续传代培育甲型肝炎病毒，并以其所得到的甲型肝炎病毒 CR326 病毒株在多种细胞系内传代增殖制备甲型肝炎疫苗的方法。美国专利 4,532,215 号和 4,636,469 号中公开了从甲型肝炎病人粪便中至少经 5 次传代以制备甲型肝炎 HM-175 病毒株的方法。美国专利，4,506,016 号公开了使甲肝病毒首先适应于人胃细胞，然后再适应于人肺纤维母细胞，以制备可用作疫苗之减毒甲肝病毒的方法。上述这些现存技术虽然分别建立了不同的方法学并分离纯化了不同的甲型肝炎减毒病毒株，但他们几乎都只是限于动物试验和小数目人体试验阶段，而且所获得病毒株的免疫接种被氧、和诱导动物抗体生成的能力远不能令人满意（如参见 P·L·Provost et al.，J·of Med·Vivol·20：165-175，1986 和 K·Midthun et al.J·of Inf·Dis·163：735-739，1991）。

中国专利申请 85107525 号公开了一株新的甲型肝炎 H_2 减毒病毒株及其纯化和减毒方法，但他们使用的病毒株与我们的不同，而且病毒株的分离条件和纯化方法也各所差异，特别是该专利申请并没有详细描述以所说的种子病毒株工业化生产甲型肝炎疫苗的方法。

本发明的一个目的是提供一种制备甲型肝炎减毒活病毒株的方法，该方法包括制备甲型肝炎急性期病人粪便样品的悬浮液，以此病毒悬浮液感染人二倍体细胞，达到病毒增殖高峰后分离细胞提取物，并以该提取物作为种子病毒来源用同一细胞基质进行连续传代减毒。

本发明的另一个目的是提供一株以上述方法制得的甲型肝炎减毒活疫苗 L-A-1 病毒株，该病毒株已于 1992 年 12 月 21 日保藏在中国典型培养物保藏中心（CCTCC），其保藏登记号为 CCTCC NoV92004

本发明的再一个重要的和更具实际意义的目的是提供一种以 L-A-1 甲型肝炎减毒病毒株为毒种，大规模工业化生产甲型肝炎疫苗的方法，该方

法包括用旋转培养法,培养人胚肺二倍体细胞并用 Earle 氏液洗细胞，然后直接接种 L-A-1 减毒病毒株，培养约 4 周后换成作为疫苗的 199 综合培养液并继续于 35-36℃下培养。

根据本发明，首先直接利用人二倍体细胞从甲型肝炎急性期病人粪便样品中分离甲型肝炎病毒颗粒，然后于低温条件下采用同一细胞基质连续传代，以得到本发明的甲型肝炎减毒活疫苗 L-A-1 病毒株，将该疫苗病毒株加到人胚肺二倍体细胞培养物中旋转培养，可大规模工业化生产甲型肝炎疫苗。

现对制备本发明甲型肝炎减毒活病毒株和以该 L-A-1 甲型肝炎减毒病毒株为毒种生产甲型肝炎疫苗的方法详细描述如下。

Ⅰ.甲型肝炎 L-A-1 病毒株的制备

1.病毒接种物的制备

采集甲型肝炎急性期病人的粪便标本，用无牛血清 Eagle 氏基本培养液（MEM）制成 5%（V/V）悬浮液，该悬浮液经高速离心后，取上清液通过 200nm 滤膜除菌过滤，所得滤液经常规血清学方法和免疫电镜观察，证实其中含有 27-32nm 大小的 HAV 颗粒，此即为病毒接种物（参见胡孟冬等，上海医学，1988，11：653-656）。

2.病毒的分离和纯化

用于分离病毒的宿主细胞为已知的人胚肺二倍体细胞系，首先将人肺二倍体细胞接种于带有玻片的小方瓶内，于 37℃下培养，培养 5-7 天后形成均匀致密的细胞单层，然后在该细胞单层上接种按步骤Ⅰ所述方法制得的病毒接种物，37℃吸附 4 小时后，补加含维生素 C 的维持液（pH7.4-7.8），再于 32-34℃下培养，培养期间每隔 1 周换液一次，以除掉不利于细胞生长的有害代谢产物，并定期用直接免疫荧光法（IF）监测细胞内病毒增殖水平，经 3-4 周后，待病毒增殖达到高峰时收集细胞，经用胰蛋白酶消化，三次反复冻融及超声处理等方法以破碎细胞，离心后得到细胞提取物，该提取物即可作为种子病毒的来源，作进一步的传代减毒。

3.病毒的传代减毒

通过在人肺二倍体细胞内连续传代，使上述强毒力病毒减毒，为此，用不含牛血清的 MEM 按

不同倍数稀释上述用作种子病毒来源的细胞提取物，接着述方法于 32℃在人肺二倍体细胞中连续传代，一般可在大约 4-5 代后转入 2BS 细胞于 37℃下继续传代，其中可每隔 5 代以蔗糖梯度法进行克隆化，共连续传 15-25 代，并于不同代次接种狨猴（Sanguinus fuscicollis）以评价减毒效果并进行体内免疫原性试验。试验表明，在连续传 10 代后病毒即已明显减毒，约 20 代时可获得令人满意的减毒效果，由此得到本发明的 L-A-1 减毒活疫苗病毒株。当最低稀释倍数（一般为 10^{-3}）的病毒接种瓶内有大约 90-100% 的被感染细胞呈现免疫荧光阳性时即可收获病毒。

该病毒株已按照专利法实施细则第 25 条的规定，于 1992 年 12 月 21 日保藏在中国武汉中国典型培养物保藏中心（CCTCC），保藏登记号为 CCTCC No.V92004。

本发明的 L-A-1 减毒活病毒株可以直接接种在适当培养基中旋转培养的人胚肺二倍体细胞单层上，于低温下培养增殖，以大规模生产完全适用于人体免疫接种的甲型肝炎疫苗。

Ⅱ.甲型肝炎减毒活疫苗的生产

首先取人胚肺二倍体细胞按 1：2-1：4 的比例扩增传代，其中细胞扩增所用培养基是添加 10-15% 小牛血清的 MEM，pH7.2-7.6，将细胞培养瓶置 37℃下旋转培养 5 至 8 天，形成均匀、致密的细胞单层，然后弃去生长液，用磷酸 Earle 氏液反复冲洗细胞 3-5 次，向培养瓶内加入适当量按前述方法制得的毒种液并于 34-36℃下培养之，每周换液一次，约 4 周后弃去维持液及残存的小牛血清，并向培养瓶内直接加入由 199 综合培养液构成的疫苗液，继续培养 2-5 天后低温冷冻收存细胞，经三次反复冻融和超声处理破碎细胞，然后离心除去细胞碎片和亚细胞结构部分，收集上清得到半成品甲型肝炎疫苗。

如此制得的半成品疫苗液，按新生物制品检定规程，经病毒滴度测定、小鼠安全性试验、牛血清含量测定、无菌试验、支原体污染检测、pH 值和外观检验、以及狨体试验等一系列检验合格后，即可作为成品甲型肝炎疫苗，用于临床进行人体或动物的预防免疫接种。

与已有技术相比，本发明生产甲型肝炎活疫苗方法的主要改进包括：（1）在病毒宿主细胞的增

殖阶段，以旋转培养法代替静止培养法，如此可显著地扩大细胞培养面积，使细胞增殖率提高5倍以上，且可以比较容易地除去或减少牛血清残存量，减小疫苗制品的不良反应。(2) 根据甲型肝炎病毒的生物学特性，用 Earle 氏液冲洗细胞表面，可更加有利于病毒的吸附感染。(3) 将疫苗液（199综合培养液）直接加入培养瓶内，可减小病毒的损失，提高疫苗的产率和滴度，并可改善病毒的稳定性。

如上文所述，在本发明 L-A-1 病毒株减毒过程中，始终以猩猩体内试验作为评价减毒效果和免疫原性的手段。因为猩猩与黑猩猩或其他灵长类实验动物相比有更好的敏感性，从而保证了毒株选育结果的可靠性和安全性。对部分产生抗体的猩猩进行攻击保护试验，发现全部有抗 HAV 的动物无论抗体滴度高或低，均可抵抗强毒的攻击，获得100%保护。

我们曾在中国国内的近 30 个地区对以猩猩为实验对象，基于 L-A-1 减毒病毒株制备的甲肝活疫苗进行了多达 10 万余人的大规模接种观察，并对其中的 3，500 人（包括对照组的 1，500 人）基于局部和全身临床反应、肝脏酶分析、血清学反应，抗体水平、粪便排毒等项指标进行了精细观察，结果表明，对不同地区、不同年龄组以多批疫苗进行的临床试用，未见有临床上有意义的不良反应。大量血清学试验结果表明，接种疫苗后可使绝大部分受试者产生良好的抗体反应，仅一次免疫注射抗体阳转率即达到 95%以上，抗体滴度 4 周 GMT 为 4.438—4.464，8 周为 5.098—6.276。

疫苗接种后进行追踪观察，未发现受试者发生再感染。在既往精细观察组中，于不同时间、不同地区随机选择部分受试者进行中和抗体和粪便排毒检测，结果表明可以产生保护性中和抗体，且粪便排毒试验均为阴性（包括使用抗原直接检测法和细胞培养法）。另外，猩猩试验还证实，凡能产生抗体反应者，无论抗体效价高低，均具有抵抗强毒株攻击的能力，

对既往接受本发明甲型肝炎疫苗的受试者追踪观察 4 年，发现抗体均持续阳性，表明该疫苗具有良好的免疫原性，免疫接种后至少可获得 4 年以上的持续保护。局部地区的流行病学调查也显示，接种该疫苗后可使易感者早期获得免疫保护，并能抵抗野生病毒的感染。

迄今我们已使用按本发明方法制得的、基于 L-A-1 疫苗株的甲型肝炎减毒活疫苗完成了近 60 万人的人体接种观察，在安全性及免疫原性方面进行了多指标详细考核，结果表明该疫苗株是极其安全和有效的甲型肝炎疫苗毒株。特别是我们首先证明了本发明的疫苗不仅具有流行病学保护效果，而且根据对乙型肝炎表面抗原（HB_sA_g）阳性志愿者的接种效果观察，首次证明了乙型肝炎病毒感染者不但可接受本发明疫苗的接种，而且可提供与健康人相同的保护作用。

实施例 1

将含有 L-A-1 病毒株的甲型肝炎急性期病人粪便样品悬浮在无牛血清 MEM 中制成 5%悬浮液，经 12，000 转／分钟高速离心 15 分钟后，取上清液用 200nm 滤膜除菌过滤，经免疫电镜观察和血清学分析表明所得滤液中确实含有 HAV 颗粒。

按常规方法培养人胚肺二倍体细胞，6 天后生长成致密的单层，向其中加入上述含病毒滤液并于 37℃下吸附 4 小时，补加维持液（pH7.6）后于 32℃低温适应传代，每周换液一次，并于不同时间用直接免疫荧光法（IF）监测病毒增殖水平，当病毒增殖达到高峰，即被感染细胞中 90%以上显现免疫荧光阳性时收获细胞，按常规方法用胰蛋白酶消化细胞，并经三次反复冻融及超声处理，以破碎细胞并提取 HAV。

同样以人胚肺细胞单层为细胞基质，按上述同样方法于 32℃下进行连续传代，传至 4 代后转入 2BS 细胞于 37℃下继续传代培养，并于不同代次进行猩猩体内减毒评价和免疫原性试验。发现在第 10 次传代后病毒即已明显减毒。将此减毒病毒株接种于同一细胞基质上继续减毒传代，并于第 20—27 代连续收获病毒，直接用于制备本发明的甲型肝炎减毒活疫苗。

实施例 2

取人胚肺二倍体细胞，添加含 10%小牛血清的 MEM（pH7.4），以 1∶4 比例在 37℃下旋转培养 7 天，使之生长成致密单层，然后弃去生长液，用新鲜配制的 Earle 氏液反复冲洗细胞表面（3 次），接种按实施例 1 所述方法制备的毒种液后于 37℃吸附 4 小时，并补加维持液（即加有维生素

C的乳白蛋白水解液）置35℃下培养，每周换液一次。4周后弃去维持液及残存的小牛血清，并直接加入199培合培养液（即疫苗液），继续培养4天后冷冻收存细胞，经三次反复冻融获得细胞。合并细胞悬液产物并以任意离心，收集上清即得到本发明的甲型肝炎疫苗。

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HEPATITIS A ATTENUATED LIVE VACCINE AND ITS PREPARATION

FIELD OF THE INVENTION

The present invention is related to a hepatitis A vaccine, in particularly, to a method for preparing hepatitis A virus strain L-A-1 and for producing hepatitis A vaccine useful for protecting human against hepatitis A in industrial scale using said virus strain.

BACKGROUND OF THE INVENTION

Hepatitis A is a worldwide distributive acute communicable disease caused by infection with hepatitis A virus (HAV). Recent reports on epidemical survey show that in developing countries or regions including China, there are as many as 4 million hepatitis A cases per year. There is frequently large-scale outbreak and rapid spread of HAV in the areas where hygiene and sanitation standards are lower, especially after various disasters. In these countries or regions, as the high incidence of hepatitis A increases, some serious public health and social problems have been encountered. Therefore, there will be an increasing need for hepatitis A vaccine which has a high specificity and safety and suitable for vaccinating the entire people.

US Patent No.4,164,566 disclosed a method for obtaining inactivated hepatitis A vaccine using hepatitis A virus strain CR326 by serial passaging in various host cells. US Patent Nos.4,532,215 and 4,636,469 described, respectively, a strain of wild-type HAV, designated HM-175, which isolated from feces of a patient, and adapted to 5 passages in African green monkey kidney culture cell and methods for obtaining a inactivated vaccine. Furthermore, US Pat. No. 4,620,978 describes a vaccine employing the HAV HM-175, triply cloned in AGMK cell culture and attenuated. These vaccines are only in their animal experiment or pro-clinical period and there are no satisfactory results were achieved (see, for example, P.L. Provost et al., J. of Med. Virol., 20:165-175,1986; K. Midthun et al., J. of Inf. Dis., 163:735-739, 19991) .

China Patent Application No.85107525 disclosed a new hepatitis A virus stain H_2. However, several distinctive purifying and lyophilizing steps was used, and no method for industrialized large quantities production of the vaccine was disclosed .

A live attenuated hepatitis A vaccine could have a significant impact on the eradication of the disease. It could be anticipated that a live attenuated vaccine which

requires minimal purification and no adjuvant would be less costly than presently available inactivated hepatitis A vaccines.

There is a need in the art for methods and HAV live vaccine or its compositions which has a high specificity and safety for effective vaccination of humans and animals against hepatitis A.

OBJECTS OF THE INVENTION

It is one object of the invention to provide a method for preparing hepatitis A live virus strain, which comprises preparing a suspension of feces of a patient suffering from acute hepatitis A, and infecting human fetal lung diploid cells (2BS), and then preparing the extracts of the cells when titers of the virus is reached to its peak value, and attenuating the viral strain by serial passaging.

It is another object of the invention to provide a attenuated hepatitis A live viral strain L-A-1 prepared by the method described as above, the sample of this strain have been deposited in China Center for Type Culture Collection under Accession No.V92004.

It is another object of the invention to provide a method for industrialized producing of hepatitis A live vaccine using the said viral strain L-A-1 as stock virus, which comprise inoculating cultivated human fetal diploid lung fibroblast cells with the resultant attenuated virus L-A-1 and cultivating the cells for about 4 weeks, and then further cultivating cells at a temperature of about 35-36 ℃.

DETAIL DESCRIPTION OF THE INVENTION

The present invention provide a attenuated hepatitis A viral strain L-A-1, and a method for producing hepatitis A vaccine by isolating hepatitis A virus particle from feces of a acute hepatitis A patient, and inoculating the purified virus on human fetal diploid lung fibroblast cells and serial passaging in the same cell substrate to obtain desired attenuated hepatitis A strain, which can be used for producing hepatitis A vaccine in industrial scale.

The present invention further provides hepatitis A virus (HAV) adapted to growth in the human fetal fibroblast cell line, 2BS, a cell substrate suitable for commercial production and licensing of inactivated and live hepatitis A vaccines. In addition to such adapted HAVs, the invention provides a method for adapting a selected HAV to growth in that human cell line and preparing an **2BS-adapted, attenuated HAV without passaging in other primate cells.** The HAV of this invention and the preparative method also preferably provides the HAV with sufficient attenuation to enable its efficacy as a vaccine for humans and animals.

Although the prior arts disclose other candidate vaccine strains of hepatitis A virus which have been adapted to growth in human diploid fibroblasts, sufficient for such adaptation have not been characterized. Thus, these strains cannot be manipulated in

vitro to assure a reproducible in large quantities and fully-characterized vaccine product.

In the method for direct isolation of HAV taken from stool samples of humans with acute hepatitis A, and further isolating and propagating the virus in a suitable substrate, the step which comprises directly passaging said virus in the **same tissue culture cells** to form a serological test or radioimmunoassay of anti-HAV.

1, preparation of hepatitis A strain L-A-1

1, preparation of viral inoculum

A new strain of HAV, L-A-1, was isolated from clinical specimens derived from an outbreak of the virus in China. Stool suspensions were prepared as 4% extracts in modified Eagle medium (MEM), pH 7.4, clarified by low speed centrifugation and removed bacteria by ultrafiltration using a 200nm filter. The resultant filtrate was tested by conventional serological and morphological observation, and it demonstrates that some hepatitis A virosome sized 27-32nm were presented therein. This stool extract was used as viral inoculum.

2, Isolation and purification of virus

Cell cultures of human fetal lung diploid fibroblast cells (2BS) were used for virus propagation. The cells were maintained in maintenance medium (Eagle's minimal essential medium, MEM) supplemented with 5% inactivated fetal bovine serum (FBS) for about 5-8 days to form a dense confluent cell monolayer in a roller bottle apparatus. The cultures were then inoculated with HAV seed virus obtained as above at a multiplicity of infection (m.o.i.) of 0.02-10. The cells were allowed a 4-hour period of absorption, after which they were again sustained in maintenance medium supplemented with ascorbic acid and cultivated at 32-34 degree C. The medium were changed and the cultures were assayed by direct immunofluorescence (IE) for hepatitis A antigen at weekly intervals. After 3-4 weeks, the cells were harvested by treatment with trypsin-EDTA and were disrupted by repeatedly freeze-thaw process when viral titers reached its peak levels. After centrifugation, the resultant extract was used as viral stock of hepatitis A virus for further attenuation.

3, Attenuation of virus by serial passaging

The obtained virulent strain were attenuated by serial passaging in the same cell substrate (the 2BS cells described as above). For this purpose, Cultures of 2BS cells were washed with the same medium (fresh eagle's medium) and inoculated with various dilution of stool material containing L-A-1 virus. The harvested infected 2BS cells were cultivated at about 32 degree C. and then the cultivated at an elevated temperature (about 37℃) by about 4-5 passges. The cell cultures were subcloned by terminal dilution at five passage intervals .

To estimate the immunogenicity of the virus strain, marmosets (Sauguirus fuscicollia) as model animal were inoculated in vivo with the L-A-1 strain of HAV prepared as above at about 10-25 passages post inoculation. The results show that the virulent strain was substantially attenuated by 10 passages post inoculation, and a satisfactory effect of attenuation was obtained by about 20 passages. A direct immunofluorescence was performed by staining with fluorescein-conjugated hyperimmune serum from a the primate species which had been infected with the L-A-1 strain of HAV. The virus were harvested when about 90-100% of infected cells inoculated with 10^{-2} dilution of attenuated virus prepared as above were positive by IF for viral antigen.

The sample of the resultant virus strain L-A-1 is available from the China Center for Type Culture Collection in Wuhan, China at December 12, 1992 under CCTCC designation number V92004.

II, production of hepatitis A live vaccine

The attenuated live virus strain L-A-1 of the present invention can be used for preparation of hepatitis A vaccine or its composition in large-scale that useful for protecting human against hepatitis A by directly inoculating a cell monolayer of human fetal lung diploid cells in a suitable medium using roller bottle apparatus, and cultivating the cells at a lower temperature.

For the purpose, cultured human fetal lung diploid fibroblast cells were proliferated by serial passaging in MEM medium supplemented with 10-15% inactivated fetal bovine serum (FBS), pH7.2-7.6. The cells were then cultivated for 5-8 days to form a dense confluent and well-distributed cell monolayer in a roller bottle apparatus. After low speed centrifugation, the medium was removed. Cultures of 2BS cells were washed 3-5 times with fresh (MEM) and inoculated with various dilution of stool material containing L-A-1 virus. The infected cells were cultivated at about 34-36℃ with medium exchange at weekly intervals. After about 4 weeks, the maintenance medium and the residual FBS were discarded, and then a 199 medium for the vaccine were directly added to culture bottle and further cultivation was conducted for about 2-5 days at a lower temperature of 32-34 degree C. Upon cultivation, the cells were disrupted by repeatedly freeze-thaw and ultrasonic treatment, and the cell debris and subcellular structures were removed by centrifugation. The supernatant so obtained as HAV stock suspension were collected.

The vaccine stock material of HAV should further be detected in term of viral titers, safeties in experimental animals, content of residual FCS, contaminant of bacteria and mycoplasmas and the like in accordance with Chinese Requirement for Bilogical Products before clinical trial in human or animals.

In method of the invention for producing hepatitis A vaccine, the improvements are following : (1) a roller bottle cultivation was take place of conventional static cultivation in period of propagating 2BS cells for substantially expanding the area of cellmonolayer under cultivation and minimizing residue of FCS; (2) Based on

biological properties of HAV, Eagle's medium was used for washing the cells for the benefit of adsorption of virus on the cells; (3) Add directly 199 medium for the vaccine into culture bottle to minimize loss of virus and to maximize the recoveries and titers of virus.

The attenuation phenotype of these viruses may be evaluated in marmosets by techniques such as described below for HAV strain H-A-1. As stated above, after a total of ten passages in 2-BS cells at reduced temperature, the resultant virus was examined for its biological characteristics in cell culture and in marmosets that are considered to be surrogates for man. The HAV H-A-1 virus was found to be temperature-sensitive (i.e., only grew at reduced temperatures) in 2-BS cells but was still capable of growing at 37.degree. In marmoset monkeys, the virus replicated poorly or not at all. This reduced capacity for replication in primates was further confirmed in human volunteers. Further, the marmosets are rendered resistant to challenge with virulent doses of hepatitis A virus.

In clinical trial, volunteers received increasing titers of the live attenuated hepatitis A vaccine which was previously tested in chimpanzees and marmosets as described above. These pre-clinical studies demonstrated that the vaccine was safe, immunogenic, and efficacious in experimental animal models.

In our clinical trials in human, about **100 hundreds healthy volunteers** living in about 30 local areas in China were vaccinated with our hepatitis A vaccine (1ml) based on the wild type live HAV strain L-A-1 ($10^{6.5}$ $TCID_{50}$/ml) from different batches. Among them, about 3,500 persons came from different regions of China were monitored and followed-up for local or systemic side effects were monitored during the admission period and for 12 weeks following the immunization. Volunteers were asked to return at 6 and 12 months for serological follow-up including aminotrasferase levels (ALT and GPT), seroconversion rates and anti-HAV titers, and virological examination of faecal extract. Stools were collected from the volunteers two to three times per week for the first 12 weeks and were tested for the presence of hepatitis A virus by radioimmunoassay. Sera were obtained prior to immunization and once a week for the next 12 weeks. In volunteers who completed the appropriate follow-up time, sera were also obtained at 6 and 12 months after initial administration of vaccine.

The results show that stools from all volunteers who received the vaccine were negative for hepatitis A virus; no local or systemic complaints were present immediately after immunization or during long-term follow-up; serum alanine aminotransferase levels remained normal in all individuals during the period of observation. Fuethermore, more than 95% of overall anti-HAV seroconversion rates were achieved after once vaccination and quantitative anti-HAV levels (Geometric Mean Titers, GMT) was about 4.438-4.464 at 4^{th} week and about 5.098-6.276 at 8^{th} week after only once vaccination.

In followed-up later for about four years, the antibody reaction were positive in all volunteers who received the vaccine of present invention, which indicate that the protective effects persists for about four years at least.

The HIV vaccine of the present invention may be used to immunize uninfected individuals from HIV infection or serve as an immunotherapeutic for those individuals already infected by HAV or by **combined HAV/HB_sA_g**. The HIV vaccine of the present invention invokes an immune response including CTLs which recognize and attack HIV infected cells and recognize the widest contingent of HIV protein. Thus, uninfected individuals are protected from HIV infection.

EXAMPLE

The following example 1 and 2 described preparation of virus strain H-A-1 and HAV vaccine, respectively.